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We have investigated phase II activation of the cooked meat mutagen 2-hydroxyamino-1-methyl-6-phenyl[4,5-*b*]pyridine (N-OH-PhIP) by cytosolic acetyltransferase, sulfotransferase, and kinase enzymes from human breast tissue. Microsomal fractions of mammary epithelial cells were also examined for prostaglandin H synthetase activation of N-OH-PhIP. The results showed that all four enzymes can participate in activating N-OH-PhIP, thereby inducing PhIP-DNA adducts in human breast cells. However, not all individuals exhibited all four activities, instead each individual showed a combination of one or more activation pathways. Mammary tissue from rats activated N-OH-PhIP exclusively by acetyltransferase catalysis and therefore may not be a good model for food mutagen metabolism in humans. Alternatively, the breast cell lines MCF-7 and MCF-10A appeared to mimic metabolic mutagen processing of mammary tumor and normal cells, respectively. The dietary compound resveratrol inhibited PhIP-DNA adduct formation in primary cultures of human mammary epithelial cells and inhibited some phase II enzymes. The present findings provide the first demonstration that the human mammary gland has the capacity to metabolically activate a dietary mutagen by multiple enzyme systems. Furthermore, these studies indicate that resveratrol may have chemopreventive properties against human breast cancer.

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INTRODUCTION

World wide breast cancer is the second most common cancer among women overall, and in developed countries, it is the most common (15,16). In the United States, in fact, breast cancer is the most common cause of cancer affecting women, the major cause of cancer deaths in women, and the major cause of death from any cause in women 35 to 54 years old (15-18). Breast cancer currently afflicts 1 of 8 women by the age of 85, accounting for an estimated 186,000 new cases and 46,000 deaths annually (17-19). Despite its high prevalence, the etiology of breast cancer is not well understood (20). Non-hereditary factors appear to dominate the etiology of breast cancer, but quantitatively important causes of breast cancer have yet to be identified. Established risk factors include radiation, full-term pregnancies, lifetime exposure to estrogen, and age (19-21). Evidence is growing that environmental exposures may be contributing to the increasing rates and geographic variation in breast cancer incidence. The question of diet and breast cancer has attracted enormous scientific and public attention because if clear links were established between breast cancer and diet, this knowledge could provide practical ways for primary prevention. It has been estimated that as many as half of all breast cancers in the United States could be related to diet (22), although this is highly speculative. Epidemiological data does show a link between breast cancer and diet (21-23). Mammary gland cancers are associated with diets rich in animal fats and meats, and the diet of Western countries typically includes a high caloric intake of cooked meats and fats. In the aggregate, however, studies indicate that dietary fat intake in adult life is not associated with breast cancer risk (23). Alternatively, it has been proposed that one dietary factor that might influence the incidence of human breast cancer is the cooked meat mutagen group composed of heterocyclic amines (24,25). Cooked meats and fish contain a number of mutagenic and carcinogenic heterocyclic amines. Although the distribution and yields of heterocyclic amines can vary with the method of cooking, alternative heating strategies do not completely eliminate cooked meat mutagens (6). Therefore, individuals who consume cooked meats in their daily diet are continually exposed to mutagenic heterocyclic amines.

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a heterocyclic amine produced during cooking of meats and fish. PhIP the most abundant heterocyclic amine in commonly fried or grilled meats and is reported to be the most prevalent of these compounds in the human diet (1,2). Metabolites of PhIP are known to form covalent adducts with DNA in many cell types including, mammary epithelial cells (6-9). The structures of 21 food-derived heterocyclic amines that form DNA-adducts are known (25,26). These compounds cause somatic cell mutations and induce cancers in animal models. Only three heterocyclic amines, however, have been shown to induce mammary gland cancer in female rodents (24,25). When added to the diet at parts per million, these agents induce mammary tumors in a dose-dependent fashion (3-5,9,27). Of the three heterocyclic amines, PhIP is the most potent mammary gland carcinogen. PhIP shows a target-organ specificity for mammary gland since it preferentially induces mammary adenocarcinomas in female rats (3-5). PhIP is regarded as being unique amongst the heterocyclic amines in its target organ specificity (14). All cooked-food mutagens tested to date induce liver cancer with the exception of PhIP (13). When maintained on a diet supplemented with PhIP, male rats preferentially developed colon cancer; whereas female rats developed mammary tumors (14,25,28). In contrast to PhIP, 2-amino-3,9-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is more representative of the imidazo classes of heterocyclic amines. MeIQx is genotoxic to liver cells and is a potent hepatocarcinogen in rodents and non-human primates (13,14). Differential metabolism of PhIP and MeIQx is apparently responsible for the tissue-specific outcomes. Although many chemicals can induce breast cancer in experimental animals, PhIP and the other two heterocyclic amines are the only mammary carcinogens known to be present in everyday diets. Therefore, of all the heterocyclic amines, PhIP is the most probable etiologic agent in breast cancer.

Heterocyclic amines require metabolic activation to form DNA adducts and exert a carcinogenic effect (29,30). Heterocyclic amine activation is a two-step process. In the first step, mixed function

oxidases convert heterocyclic amines to N-hydroxylamine metabolites via liver microsomal cytochrome P450 enzymes (10,11,31-34). The N-hydroxylamine intermediates are further activated by phase II enzymic esterification reactions to generate the ultimate reactive forms which exhibit tissue-specific DNA adduct formation (35-38). PhIP, like many other carcinogens, does not react with DNA until it undergoes metabolic transformation. Human microsomes are reported to have a high capacity to metabolize PhIP to the proximal mutagen N-hydroxy-PhIP (N-OH-PhIP)(39-41). The second step or phase II activation is an esterification reaction that occurs in the target cell, i.e. it is assumed that the target cells activate the N-hydroxylamine metabolite to the ultimate mutagen. The initiation of mammary cancer could be related to the ability of mammary gland cells to transport and metabolize heterocyclic amines or their metabolites. Little is known, however, about the metabolic processing or genotoxic effects of heterocyclic amines in human mammary gland epithelial cells. Normally, metabolism of food-derived heterocyclic amines is carried out by cytochrome P450 and other enzymes in the liver, but other tissues have the potential to metabolize heterocyclic amines (11,12). The initiation of dietary-related breast cancer could be related, in part, to the ability of mammary epithelial cells to transport and metabolize heterocyclic amines (or their metabolites) to genotoxic species. When our work was initiated, little was known about the metabolic processing of PhIP and other heterocyclic amines in human mammary epithelial cells. Such studies are required to identify the enzymes responsible for metabolic activation of these compounds, to characterize the carcinogen processing in mammary epithelial cells, to determine preferential genotoxic effects of heterocyclic amines in these cells, and to examine the ability of mammary gland cells to repair their genome.

There are several possible routes of N-hydroxy heterocyclic amine phase II activation in human cells. These phase II activation pathways include acetyltransferases, sulfotransferases, tRNA synthetases/kinases, and hydroperoxidases. Mutagen activation in mammalian cells via tRNA synthetases/kinases has scarcely been examined and thus is not well documented. Alternatively, mammalian acetyltransferases and sulfotransferases have been extensively studied. Activation by arylamine N-acetyltransferases (NATs) is a major route in the metabolism of numerous drugs and carcinogens, including heterocyclic amines. NAT also functions as an O-acetyltransferase for N-hydroxy heterocyclic amines formed by CYP1A2. Humans have two highly similar genes encoding NAT1 and NAT2 enzymes that catalyze these reactions. NAT1 and more recently NAT2 have been shown to be polymorphic enzymes that give rise to rapid and slow acetylator phenotypes in the population (54,55,72). One or more NAT and CYP1A2 polymorphisms might be an important determinant of cancer risk associated with environmental and dietary agents. For example, Lang et al. (73) found that rapid acetylators and rapid N-hydroxylators are disproportionately represented in colon cancer cases, and this risk increases substantially for individuals who frequently eat well-done red meats. A similar paradigm has not been established for breast cancer. Recently, Sadrieh et al. (55) reported that human mammary gland has NAT1, but not NAT2, catalytic activity, and NAT1 protein was capable of metabolically activating heterocyclic amine food mutagens. As described in the Results, we have now shown that N-OH-PhIP acetylation is a primary pathway for phase II activation in human breast tissue.

In humans, three major cytosolic sulfotransferases have been identified: two isozymes of phenol sulfotransferase and a steroid/bile acid sulfotransferase also referred to as dehydroepiandrosterone (DHEA) sulfotransferase (70). The two phenol sulfotransferase (PST) can be distinguished from each other on the basis of thermal sensitivity and resistance to 2,6-dichloro-4-nitrophenol (DCNP) (70). Carcinogenic arylamines and heterocyclic amines are substrates for the human PSTs. In fact, N-OH-PhIP is a substrate for both the thermal labile (TL-PST) and the thermal stabile (TS-PST) human isozymes. Chou et al. (71) showed 3'-phosphate-adenosine-5'-phosphosulfate (PAPS)-dependent DNA binding activity of N-OH-PhIP in human liver and colon cytosols but not in pancreas, larynx, or urinary bladder epithelial cytosols. DNA binding of the N-hydroxyl derivative correlated with levels of TS-PST

but not the other two sulfotransferases. This and other data suggested that TS-PST is expressed polymorphically in human populations and may play a role in determining interindividual susceptibility to dietary carcinogens (70,71). Our studies are the first to demonstrate sulfotransferase activation of N-OH-PhIP in human breast tissue cytosols. Prostaglandin H synthase (PHS), an arachidonic acid-dependent peroxidase, is known to be present in the microsomal fraction of several tissues. PHS is a bifunctional enzyme that initiates prostaglandin biosynthesis through oxidation of arachidonic acid (AA). Some chemical carcinogens, including aromatic amines, function as reducing cofactors for the peroxidase and thereby undergo peroxidative metabolism. The resultant oxidized amine can react with DNA. The results presented below are also the first demonstration of PHS activation of dietary heterocyclic amines in human breast samples

Strategies to control breast cancer should include primary prevention based on understanding the causes of breast cancer (42). While changes in diet and limiting exposure may someday alter breast cancer incidence, chemical intervention offers an attractive approach for more immediate results. One goal of chemoprevention is the identification of safe and effective agents for the prevention of cancer (42,43). After identifying candidate agents, a second objective is to determine their mechanisms of action which would facilitate development and clinical testing. General classifications of chemopreventive activity include antiproliferatives, antioxidants, and phase I or phase II blocking agents (43). Knowledge of mechanisms of action can be used to rank agents for use and to design multiple agent protocols. Therefore, it is highly desirable to understand the mode by which chemicals delay or prevent cancer.

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a phytoalexin found in over 70 different plant species, many of which are components of the human diet, such as peanuts and mulberries (44). Relatively high quantities of resveratrol are found in fresh grape skin and thus red wines are enriched for this compound. Resveratrol is of interest because of its potential for chemopreventive activity in a variety of cancers and because of its apparent low toxicity. Furthermore, it is already a common dietary component. Resveratrol may have chemopreventive activity via multiple mechanisms. Resveratrol was reported to act as an antioxidant and antimutagen (45) and to induce drug metabolizing enzymes, such as quinone reductase which can detoxify carcinogens (46). Resveratrol inhibited cyclooxygenase and hydroperoxidase activities associated with cell signaling pathways of inflammation and proliferation (47). In other studies, resveratrol induced differentiation of HL-60 leukemia cells to a non-proliferative phenotype, showing antiprogression activity. More directly, resveratrol inhibited tumorigenesis in the two-stage DMBA/TPA mouse skin cancer model and retarded the development of preneoplastic lesions of carcinogen-treated mammary gland in culture (47). In this report, we present evidence that resveratrol has anti-initiation activity with human mammary epithelial cells. As shown in the Results section, resveratrol inhibited the formation of PhIP-DNA adducts in primary cultures of human breast cells exposed to the proximal mutagen N-OH-PhIP. We have now examined several potential mechanisms responsible for this inhibition.

In conclusion, the studies presented here have direct relevance for human breast cancer because human daily exposure levels to PhIP are estimated to be comparable to those of environmental carcinogens such as N-nitrosodimethylamine and benzo[a]pyrene (24). DNA damage in the mammary gland caused by daily exposure to heterocyclic amines could be accumulatively fixed as mutations. Since human breast cancer cells contain multiple genetic alterations and carcinogenesis requires a number of steps (48), it is possible even low levels of these dietary mutagens could play a role in one or more of these steps and thus, contribute to the incidence of dietary-related human breast cancers. Changes in diet and limiting carcinogen exposure may someday alter breast cancer incidence, and strategies to control breast cancer should include primary prevention based on understanding the causes of breast cancer (42).

BODY

EXPERIMENTAL PROCEDURES

Human Breast Tissue and Human Mammary Epithelial Cell (HMEC) Culture. Human breast tissue was obtained as residual surgical material from reduction mammoplasties of healthy donor women through the Cooperative Human Tissue Network or as excess pathology tissue from mastectomies of cancer patients at the University of South Alabama Hospitals and Clinics. Tissue was collected aseptically in the operating room and transported on ice to the laboratory. Tissue was grossly dissected, and disassociated fat and connective tissue was discarded. The remaining tissue, composed mostly of mammary parenchyma with some associated dense connective tissue and white fat, was finely minced and incubated overnight at 37° in 5 ml/g wet weight of Tyrode Solution containing 1 mg/ml of collagenase (type I), 150 μg/ml of hyaluronidase, 10% fetal calf serum (FCS), and 10 μg/ml of DNase I (61). Tissue suspensions were centrifuged and washed with DMEM media to remove fat. Pellets containing mammary epithelial and stromal cells were diluted with DMEM media containing penicillin (100 U/ml) and streptomycin (100 μg/ml) and filtered through a 53 μm filter according to Stampfer (61). This procedure traps most of the ductal fragments containing clusters of epithelial cells on the filter, whereas the monodispersed stromal cells and blood cells will pass through the filter. The retained mammary ductal fragments were rinsed, collected by reverse washing of the filter, and plated into primary culture. Cells were suspended in DMEM supplemented with 1% FCS, 10 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For longer-term cultivation of HMEC, serum-free MEGM medium (Clonetics, San Diego, CA) is used as described previously (8). Cell lines MCF-7, MCF-10A, and ZR-75-1 were purchased from ATCC and were grown, passaged, and frozen according to the ATCC's recommendations. Before cells are exposed to a food mutagen, the culture media was removed, and cells were washed with warm PBS. Cells were exposed to a heterocyclic amine, either the parent compound or the N-hydroxylated species that had been mixed with medium minus serum and antibiotics. After exposure, the mutagen/media mixture was removed, and the culture was again washed with PBS. Cells were either collected immediately by trypsin treatment or complete media was added back to the cultures for cytotoxicity or genotoxicity studies.

Synthesis of N-hydroxylamine Metabolites of MeIQx and PhIP. MeIQx and PhIP were purchased from Toronto Research Chemicals, Inc.(Toronto, Canada). Attempts to synthesize N-hydroxy-MeIQx and N-OH-PhIP are discussed under the Results section. The 2-nitro analogs of MeIQx and PhIP were prepared by the method of Gravis (63), then N-OH-PhIP and N-OH-MeIQx were to be synthesized by reduction of the nitro derivatives following the methods of Lin et al. (64). Reaction products were purified by reversed-phase HPLC on C_{18} semi-prep columns. Chemical identities were established by Fast Atom Bombardment-mass spectra generated on a VGZAB2SE double-focusing mass spectrometer in the positive-ion mode, employing xenon atoms accelerated to 9-10 kV.

Cytotoxicity Studies. Lactate dehydrogenase (LDH) leakage from cells was used as a biomarker for cellular damage. LDH activity released into the medium was determined as a function of mutagen concentration and treatment/post-treatment time. Media was removed and triplicate assays were performed for each culture. Oxidation of lactate to pyruvate was assayed according to Cabaud and Wroblewski (66). The results are expressed as a percentage of total LDH activity that is released into culture media. LDH leakage from HMEC of greater than 15% of total LDH activity was be judged overtly toxic to cells, and such cells were not used for experiments.

Preparation of Cytosolic and Microsomal Fractions. Human breast tissue was minced with scissors in a small beaker and sufficient STM buffer (0.25 M sucrose, 10 mM triethanolamine, pH 7.4, and 5 mM β-mercaptoethanol) was added to cover the tissue. This suspension was transferred to a 30-ml Corex

centrifuge tube and homogenized with short bursts of a Polytron set on #5 until the tissue was completely disrupted. The homogenate was centrifuged at 105,000 x g for 1 h at 4°. The resultant supernatant was used as the cytosolic fraction. The pellet was used as the microsomal fraction. Protein concentrations were determined by the method of Lowry et al. (69) using BSA as a standard.

Determination of Sulfotransferase Activity. PAPS-sulfotransferase activation of N-hydroxylamine heterocyclic compounds was assayed by a method modified from Lin et al. (50). Incubation mixtures consisted of 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mg calf thymus DNA, 0.5 - 1.0 mg cytosolic protein, 0.2 mM PAPS in a total volume of 0.2 ml. Assay mixtures were purged with argon and warmed at 37° before the addition of substrate. The reactions were initiated by the addition of 20 μM N-OH-PhIP. Substrate was added in 5 μl of DMSO-ethanol, 4:1, using a 2 mM stock solution of [^{3}H]-N-OH-PhIP. The head space was filled with argon and assay mixtures were incubated at 37° for 30 min. Control reactions either did not contain PAPS or cytosolic protein, and control values are subtracted from experimental samples. Reactions are terminated by the addition of 2 vol of water-saturated n-butanol. The aqueous phase was made 300 mM sodium acetate (1/10 vol of 3 M stock), mixed well, and extracted twice with buffer-saturated phenol-chloroform (1:1), and the DNA was precipitated with 2.5 vol of cold ethanol. Precipitated DNA was taken up in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The concentration of DNA recovered from the reaction was determined from A_{260} readings or by the diphenylamine colorimetric test or both. The extent of covalent binding was determined by ^{32}P-postlabeling assays as described below.

Determination of Acetyltransferase Activity. Acetyl-CoA-dependent N-OH-arylamine O-acetyltransferase activity was determined as reported by Lin et al. (50). The assays were performed for 30 min at 37° in argon-saturated 50 mM pyrophosphate buffer (pH 7.4) containing 1 mM DTT, 1 mM acetyl CoA, 2 mg/ml calf thymus DNA, 1-2 mg/ml cytosolic protein, 0.1 mM EDTA, and 20 μM of N-OH-heterocyclic amine substrate. The reactions were assembled on ice and initiated by placing them at 37° in tightly sealed tubes with argon saturation of incubation mixture and head space. The DNA-binding assays were terminated by the addition of 2 vol of water-saturated n-butanol. The DNA was isolated by sequential extractions with n-butanol, followed by chloroform-phenol extractions and by ethanol precipitation in the presence of 300 mM sodium acetate. Mutagen-DNA binding was determined by the same methods as those used for sulfotransferase activation of heterocyclic amines. The non-enzymatic binding of N-OH-heterocyclic amine to DNA was estimated by omitting acetyl CoA from the incubation mixture. Reaction conditions were saturating for substrate, for acetyl CoA, and first order with respect to protein concentrations.

Determination of Aminoacyl-tRNA Synthetase and ATP-Dependent Kinase Activation. Cytosolic L-proline-dependent and ATP-dependent tRNA synthetase and ATP-dependent kinase activities were determined similar to methods used by Davis et al.(35). Incubation mixtures of 0.2 ml contained 50 mM potassium N,N'-bis(2-hydroxyethyl)glycine (Bicine) buffer, pH 8.0, 2 mg/ml calf thymus DNA, 1 mM DTT, 3 mM magnesium acetate, 1 mM L-proline and/or 1 mM ATP, 20 μM [^{3}H]N-OH-HA, and 1 mg/ml of cytosolic protein. There were three assays: (1) cytosol plus proline (**L-proline-dependent activation**), (2) cytosol plus ATP (**ATP-dependent kinase activation**), and (3) cytosol plus proline and ATP (**ATP-dependent tRNA synthetase activation**). Control reactions had no cytosol. Incubations were at 37° for 30 min and were terminated by adding 2 vol of water-saturated n-butanol, and the DNA is isolated by standard procedures.

Preliminary data thus far indicate that human breast tissue does not have the ability to activate N-OH-PhIP via L-proyl-tRNA synthetase catalysis. On the other hand, kinase activation of the mutagen appears to be a significant pathway for DNA adduct formation in mammary gland cells of some individuals - cancer patients in particular. To our knowledge, we are the first laboratory to begin

characterizing this ATP-dependent activation, and this work is at a very early stage. As described in our report, the reaction is ATP-dependent, requires cytosolic protein which can be inactivated by heating, and evidently requires ATP-hydrolysis since it is inhibited by γ-S-ATP.

Determination of Prostaglandin H Synthase Activity. The assay used was modified from Flammang et al. (76). Assays (0.2 ml) contained 50 mM potassium phosphate buffer (pH 7.4), 100 μM arachidonic acid, 2 mg/ml calf thymus DNA, 20 μM N-OH-PhIP, and 1 mg/ml microsomal proteins. Mixtures were preincubated at 37° for 2-3 min, and the reactions were started by the addition of arachidonic acid. Arachidonic acid stock solutions are prepared fresh daily (10 mM in argon-purged ethanol). Control reactions without AA or without protein were also carried out. In a separate reaction, 20 μM indomethacin was added as an inhibitor of PHS. A 10 mM stock solution of indomethacin in DMSO was prepared and stored at -4°C. Another reaction assayed the ability of HMEC microsomes to activate the parent mutagen, e.g., PhIP. The reactions were incubated for 30 min at 37° and then terminated by the addition of an equal vol of water-saturated butanol. The DNA was isolated by standard methods. Our results are the first to show N-OH-PhIP activation by PHS from any tissue or cell type. There is one problem with performing this assay using mammary gland tissue because of insufficient microsomal protein obtained from the amounts of breast tissue that is routinely provided to us.

DNA Isolation. Mammary epithelial cells were treated with heterocyclic amines or the N-hydroxylamines as discussed above. After carcinogen treatment, cells were lysed with 10 mM Tris, 1 mM EDTA, pH 8.0 (TE buffer) containing 0.5% SDS. Proteinase K was added to 50 μg/ml and the lysate was digested overnight at 39°. The ionic strength of the solution was increased to 150 mM NaCl, the digest was extracted twice with equal volumes of buffer-saturated chloroform:phenol (1:1), and the nucleic acids precipitated with ethanol. Pellets were dried and dissolved in TE buffer, RNases A and T1 were added to 50 μg/ml and 100 units/ml, respectively, and incubated for 1 h at room temperature. One-tenth volume of 3 M sodium acetate, pH 5, was added and the solution was extracted several times with chloroform:phenol. DNA was recovered from the aqueous phase as an ethanol pellet. Isolated DNA was characterized as described in our other work (82,83).

^{32}P-Postlabeling. DNA was digested with micrococcal nuclease and phosphodiesterase II to obtain deoxyribonucleotide-3'-monophosphates. Nucleotides were labeled by the standard method described by Gupta et al. (52). Briefly, 0.17 μg of digested DNA was labeled with 0.6 nmol of [γ-^{32}P]ATP (600 Ci/mmol) in 10 μl of kinase buffer by 2.5 units of T4 polynucleotide kinase for 30 min at 37° to produce [5'-^{32}P]deoxyribonucleoside-3',5'-bisphosphates. To determine total nucleotide labeling for each sample, 1 μl of the labeling reaction was removed and rapidly mixed with 59 μl of 10 mM Tris-HCl, 1 mM EDTA, pH 9.5. Five μl aliquots of this dilution were applied to a TLC-sheet and chromatographed with 0.8 M ammonium formate, pH 3.5 (52).

^{32}P-Mapping. Following labeling, normal nucleotides were separated from modified nucleotides by TLC development of PEI-cellulose sheets with 1.7 M sodium phosphate, pH 6.0. Modified nucleotides remained at the origin while normal nucleotides migrate away from the origin (52). The modified nucleotides were contact-transferred to another PEI-cellulose sheet and were developed with the following solvent systems: (D1, first dimension) 3.6 M lithium formate - 6.8 M urea, pH 3.5; (D2, second dimension at right angles to D1) 1.0 M lithium chloride - 0.5 M Tris-HCl - 8.5 M urea, pH 8.0; followed by redevelopment in D2 with 1.7 M sodium phosphate buffer to reduce background radioactivity. Adducts were detected by autoradiography on X-ray film with intensifying screens at -70°C. Using this TLC separation system, we have observed one abundant adduct and 2 minor adducts in HMEC DNA of cultures exposed to N-OH-PhIP. No adducts above background have been noted for experiments using the parent compound.

Adduct Quantification. DNA adducts were detected by autoradiography. The radioactivity associated with a modified nucleotide was determined as described by us in previous work (77,78). Relative Adduct Labeling (RAL) and adduct quantification was carried out by the method of Schut and Herzog (53).

DNA Repair Assay. To uniformly label DNA, mammary epithelial cells (10^6 to 10^7 cells) were cultured in the presence of 5 μCi/ml ^{3}H-thymidine and 10 μM thymidine until they became confluent (79). Labeled cells were washed with warm PBS and treated with carcinogen for 2 h. Control cultures were not exposed to carcinogen. Cells from the control cultures and those of 0 h repair (initial damage levels) were lysed immediately. For repair analyses, the mutagen was removed, cell were thoroughly washed with PBS, and the cultures are replenished with fresh culture media containing 10 μM bromodeoxyuridine and 1 μM fluorodeoxyuridine to density label newly synthesized DNA. At 8, 24 and 48 h, cells were lysed. Parental DNA was separated from replicated DNA on neutral CsCl gradients as described by Bohr and Okumoto (79). Fractions containing non-replicated DNA were pooled and dialyzed versus TE buffer overnight. DNA isolated from these fractions was subjected to adduct analysis as discussed under postlabeling methods.

RESULTS AND DISCUSSION

Synthesis of N-Hydroxy-Heterocyclic Amine.

N-OH-MeIQx and N-OH-PhIP are prepared from the nitro derivatives of the parent compounds (64). A typical synthesis is carried out as follows: 50 mg of MeIQx was dissolved in 6.4 ml of N,N-dimethylformamide/acetic acid (1:1). This was added drop-wise over a period of 15 min to a solution of 2.35 g of sodium nitrite in 5 ml of water to produce NO_2-MeIQx which precipitated out of solution as it formed (63). The precipitate was washed several times with 100 ml of cold water. The precipitate was first air-dried, then vacuum-dried and dissolved in methanol. Ultraviolet absorbance showed a spectra with a maximum at 305 nm and a shoulder in the vicinity or 350 nm. There was no indication of a peak in the 270 nm to 275 nm range (wave length of absorbance maxima for parent, MeIQx) and in fact this region was very close to the bottom of a trough in the spectrum. Therefore, it appears the parent compound was completely derivatized to NO_2-MeIQx, and the yield was approximately 80%.

N-OH-MeIQx is obtained by the reduction of the nitro compound with hydrazine and palladium on charcoal. Briefly, 2.43 mg (10 μmol) of nitro-MeIQx was dissolved in 5 ml of tetrahydrofuran and 5 mg of palladium on charcoal was added while stirring vigorously; this was set in an ice-salt bath. After cooling on ice for a minimum of 10 min, 20 μl of hydrazine hydrate was added and the mixture was stirred under argon for 30 min. The reaction was terminated by dilution of the mixture 10-fold with argon-saturated 10 mM EDTA, pH 4.8. The resultant N-OH-MeIQx was isolated by application to a C_{18} Sep-Pak (500 mg cartridges) with chromatography as described by Lin et al. (64). The result indicated that the reaction was complete because the absorbance spectra had a peak at 275 nm with a trough at 305 nm (34). A standard curve of N-hydroxylamines suggested that 1 mg or 40% of the starting material was recovered. However, FAB mass spectrometry in the positive ion mode did not show a m/z 229 mass expected for N-OH-MeIQx. Instead a peak at 214, corresponding to a MH^+ of the starting compound MeIQx, was observed. This result implied that the nitro derivative had been over reduced. We spent several months varying the ratios of NO_2-MeIQx to palladium on charcoal and to hydrazine hydrate and varying the reaction times and temperatures without substantially improving the yield of N-OH-MeIQx. An alternative method of N-OH-MeIQx synthesis via ascorbic acid reduction of nitro-MeIQx (34) was used in several attempts to make the proximal mutagen but without success.

A reviewer of our grant application had informed us that N-OH-MeIQx and N-OH-PhIP could be purchased from the NCI Chemical Carcinogen Reference Standard Repository at Midwest Research

Institute (Kansas City, MO). As soon as this grant was funded, we placed orders for both N-OH-heterocyclic amines (08/01/96) with Midwest Scientific. These N-hydroxylamines are unstable and are only synthesized when sufficient quantities of carcinogen are ordered at $300 per 5 mg. We received our first shipment of N-OH-PhIP on 10/09/96 which has since been refilled twice more. We have not yet received any N-OH-MeIQx. We were told that the government contractor had attempted to synthesize some and had failed. We increased the amount of our original order on 07/30/97. We will probably cancel this order very soon since there is not much time left on this grant to do the MeIQx experiments. We do not know why the synthesis described above did not work which is perplexing because we have used the protocol in the past with success.

Human Breast Tissue Has Multiple Pathways for Activating the Food-Derived Mutagen N-Hydroxy-PhIP.

Initiation of carcinogenesis by heterocyclic amine compounds is believed to be due to their adduction to DNA following metabolic activation of the parent amines. As shown in Figure 1, the first step in the bioactivation of PhIP is a cytochrome P450-mediated hydroxylation of the amine nitrogen, catalyzed by liver microsomal P450 1A2 in humans (39). To react with DNA and form specific adducts, N-hydroxy-PhIP (N-OH-PhIP) must be esterified by one or more enzymes during phase II activation. Esterification of N-OH-PhIP is low in human liver tissue with relatively few PhIP-DNA adducts produced in this organ (49). Therefore, N-OH-PhIP is released systemically for potential phase II activation in extrahepatic tissues, such as breast tissue, wherein these activation processes could play a significant role in organ-specific tumorigenesis. Figure 2 shows all known phase II activation reactions for N-hydroxy-heterocyclic amines.

To determine what phase II systems can metabolically process N-OH-PhIP to PhIP-DNA adducts, we have tested for acetyltransferase, sulfotransferase, and aminoacyl-tRNA synthetase/kinase activation by human breast tissue cytosol. In addition, microsomal fractions of mammary epithelial cells from some individuals were examined for prostaglandin H synthetase activation of N-OH-PhIP. Human mammary gland tissue, removed from healthy women undergoing reduction mammoplasty, was obtained from The Cooperative Human Tissue Network. Residual surgical breast tissue was also obtained following mastectomy from the University of South Alabama Hospitals. Cytosolic and microsomal fractions were prepared by standard differential centrifugation. Enzyme assays were performed immediately after cytosol isolation as described (50). The assay mixtures (0.2 ml) contained 100 mM potassium phosphate (pH 7.4), 1 mM DTT, 2 mg/ml calf thymus DNA, 1 mg/ml cytosolic protein, 0.1 mM EDTA, and 20 μM N-OH-PhIP. Enzyme assays and concentrations of enzyme-specific cofactors were: acetyltransferase, 1 mM acetyl-CoA; sulfotransferase, 0.2 mM 3'-phosphoadenosine 5'phosphosulfate, and; tRNA synthetase/kinase, 1 mM L-proline and 1 mM ATP. The sulfotransferase assay did not contain DTT, and the kinase assay had 50 mM Bicine (pH 8.0) in place of potassium phosphate. To measure PHS peroxidase activation, assays contained 50 mM potassium phosphate buffer (pH 7.4), 100 μM arachidonic acid, 2 mg/ml DNA, 20 μM N-OH-PhIP, and 1 mg/ml microsomal protein. In separate reactions, 10 μM indomethacin was added as an inhibitor of PHS (51). Enzyme-specific cofactors were absent in negative control assays. Assay mixtures were purged with argon, substrate was added, and mixtures were incubated at 37° for 30 min. The reactions were terminated and modified DNA isolated as described (50). ^{32}P-postlabeling analysis was performed as reported earlier using the ATP-deficient method (52). PhIP-DNA adducts were detected by autoradiography and quantified by the method of Schut and Herzog (53).

The ^{32}P-postlabeling method was used to test the ability of human breast tissue enzymes to convert the intermediate mutagen N-OH-PhIP to PhIP-DNA adducts. This in vitro phase II metabolic activation of N-OH-PhIP was measured by the binding of PhIP to exogenous DNA in the presence of human breast tissue cytosolic or microsomal proteins and specific cofactors for acetyltransferase, sulfotransferase, tRNA synthetase\kinase, and PHS. Figure 3 is a composite of autoradiograms showing

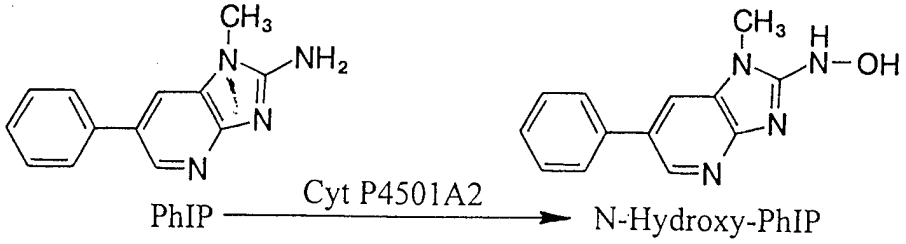

FIGURE 1. The chemical structure of the cooked meat promutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is shown on the left side. During phase I activation, PhIP is oxidized in human liver tissue by the microsomal enzyme cytochrome P450 1A2 to form N-hydroxy-PhIP, an intermediate or proximal mutagen, shown on the right side.

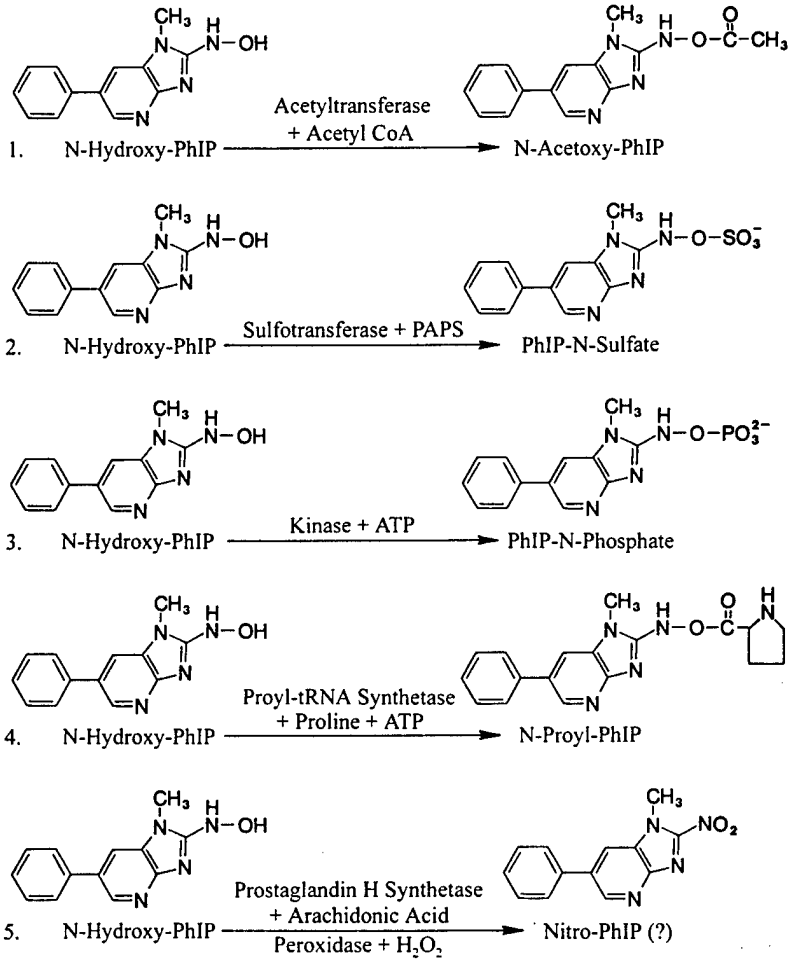

FIGURE 2. Proposed phase II activation pathways in human mammary gland epithelial cells for the metabolic conversion of N-hydroxy-PhIP to compounds capable of reacting with DNA. The enzyme, cofactor(s) and reactive PhIP species is shown in each of the 5 reactions. PAPS is adenosine 3'-phosphate 5'-phosphosulfate. In pathway number 5, prostaglandin H synthetase or another cellular peroxidase could catalyze the oxidation reaction. The putative nitro-PhIP product is not well characterized.

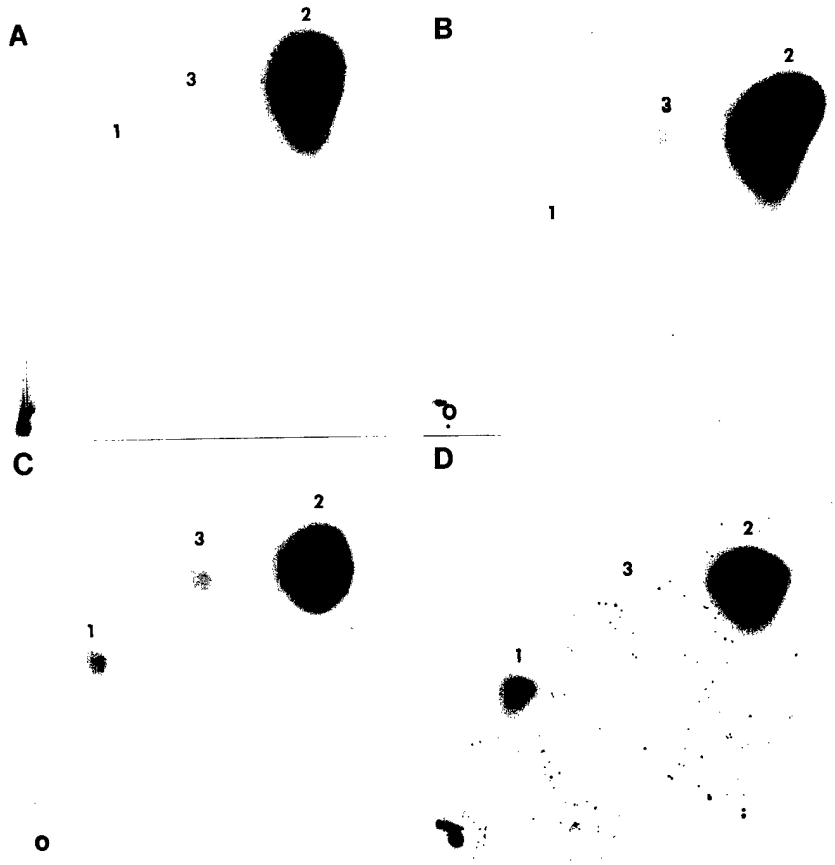

FIGURE 3. Autoradiograms showing PhIP-DNA adduct patterns obtained by incubating 20 μM N-OH-PhIP with 2 mg/ml of calf thymus DNA in the presence of: (A) 1 mM acetyl CoA; (B) 1 mM ATP; (C) 0.2 mM PAPS, and; (D) 100 μM arachidonic acid.

mutagen activation by protein fractions of human breast cells. The data demonstrate that human mammary gland has the capacity to metabolically activate N-hydroxy food mutagens through at least four different enzyme pathways, thereby inducing PhIP-DNA adduct formation in human mammary cells. The results obtained with samples from 18 donors are listed in Table 1. Although 3 cytosolic and 1 microsomal enzyme systems can participate in esterifying N-OH-PhIP, not all individuals exhibited all these activities, but instead each donor showed a variable combination of one or more of these activities. For example, donor number 25 showed N-OH-PhIP activation via all 4 esterification reactions, whereas only acetyltransferase and PHS peroxidase from donor number 26 activated the mutagen. Thus, each individual exhibited a unique activation profile.

To validate the in vitro system used here, we compared adduct patterns generated by primary cultures of mammary epithelial cells and by the enzyme assays described above. Three PhIP-DNA adducts are induced in cultured human mammary epithelial cells exposed to N-OH-PhIP (8). The same three PhIP-DNA adducts were also observed via ^{32}P-postlabeling following all esterification assays that resulted in DNA binding (data not shown). In addition, we repeatedly failed to detect adducts generated with the parent compound PhIP (20 to 100 μM) either in the enzyme assays or in cultured cells. This latter result indicates that PhIP phase I enzymes are absent or very low in human mammary epithelial cells.

The results in Table 1 suggest that acetyltransferase and tRNA synthetase/kinase are major pathways of phase II activation, whereas sulfotransferases and PHS play less prominent roles in N-OH-PhIP esterification. Of the four phase II transferases in human breast tissue, acetyltransferase and tRNA synthetase/kinase had the highest capacity to induce PhIP-DNA adducts. Positive results of sulfotransferase and PHS peroxidase gave uniformly lower levels of PhIP-DNA adducts, ranging from 0.1×10^{-6} to 2.3×10^{-6} adducts/nucleotide. Sixteen out of eighteen samples had detectable acetyltransferase activity; 10 with levels of PhIP-DNA adducts greater than 10^{-5} adducts/nucleotide and 8 with lower levels. We assume that this grouping corresponds to the bimodal distribution of rapid and slow acetylators representing the polymorphic N-acetyltransferase loci of the human population (54) with the 2 not detected (ND) samples being on the lower end of the slow acetylators. Sadrieh et al. (55) have observed a similar distribution of N-acetyltransferase expression and activity in the human mammary gland. In contrast to the acetyltransferase results, only two of the samples showed tRNA synthetase/kinase RAL activity greater than 10^{-5}. In the preliminary studies, experiments did not distinguish between tRNA synthetase activation and kinase activation because control assays lacked only ATP which would be required for both enzymes. However, subsequent work described in a section below, showed that tRNA synthetase was not activating the proximal mutagen, and DNA-PhIP adducts were generated solely by an ATP-dependent activity. This and other experiments suggested that a kinase or kinases were responsible for esterifying N-OH-PhIP. A preliminary report describing these results is in press in the journal Nutrition, and preprints are included in the **APPENDIX**.

In conclusion, the present findings provide the first demonstration that the human mammary gland has the capacity to metabolically activate a dietary mutagen by alternative enzyme systems, including acetyltransferase, sulfotransferase, tRNA synthetase/kinase and prostaglandin hydroperoxidase catalysis. Individual phase II activation patterns ranged from high acetyltransferase activity, low sulfotransferase activity, and no detectable kinase activity to high kinase activity, low acetyltransferase and sulfotransferase activities. Therefore, human mammary gland cells possess multiple N-OH-PhIP activation systems that display highly variable interindividual levels of mutagen processing enzyme activities. In vivo, such processes may play a role in the initiation of breast cancer. Additional studies will be required to determine if any of these enzyme activities, or combination of activation pathways, constitute a risk factor for mammary tumors in humans.

A Kinase-Like Activity from Human Breast Tissue Can Activate N-OH-PhIP.

An ATP-dependent type of cooked food mutagen activation was described a number of years ago

Table 1. N-Hydroxy-PhIP Binding to DNA Mediated by Human Breast Tissue Phase II Activating Enzymes.

Donor Number	Tissue[a] Source	Age	Ethnic[b] Origin	PhIP-DNA Adducts (RAL x 10^{-6})[c] Mean ± S.E. (N)[d] Acetyltransferase	Sulfotransferase	tRNA Synthetase/Kinase[e]	Peroxidase[f]
21	RM	39	CA	33.0 ± 4.7 (3)	1.2 ± 0.2 (2)	ND[g] (2)	NA[h]
25	NTB	58	AA	1.6 ± 0.3 (2)	2.3 ± 0.8 (2)	21.6 ± 3.5 (2)	1.5 ± 4.1 (2)
26	RM	37	CA	20.6 ± 6.3 (3)	ND (3)	ND (3)	1.6 ± 0.3 (2)
27	TNA	48	CA	1.3 (1)	0.5 (1)	3.7 (1)	NA
28	RM	22	CA	17.8 ± 2.7 (3)	0.7 ± 0.2 (3)	0.6 (1)	ND (1)
29	RM	33	AA	ND (2)	NA	3.8 ± 0.1 (2)	NA
30	TNA	50	CA	ND (2)	1.0 ± 0.6 (2)	2.6 ± 0.3 (2)	NA
37	TNA	84	AA	2.2 ± 1.1 (2)	1.0 (1)	0.7 ± 0.2 (3)	NA
49	RM	26	CA	18.9 ± 7.8 (3)	0.4 ± 0.2 (2)	0.3 ± 0.1 (3)	NA
57	TT	73	AA	14.4 ± 5.6 (2)	0.2 (1)	12.8 (1)	0.1 ± 0.02 (2)
71	RM	23	CA	20.7 ± 5.5 (2)	1.0 ± 0.4 (2)	ND (2)	NA
74	RM	21	CA	8.2 ± 0.3 (2)	ND (2)	0.2 ± 0.1 (2)	NA
77	TT	37	AA	16.3 ± 6.5 (3)	0.2 ± 0.1 (3)	0.5 ± 0.1 (3)	0.2 ± 0.03 (3)
78	TT	41	AA	19.1 ± 3.6 (3)	0.4 ± 0.1 (3)	0.2 ± 0.1 (3)	0.1 ± 0.05 (3)
80	TT	35	CA	9.3 ± 2.7 (3)	0.2 ± 0.1 (3)	0.3 ± 0.1 (3)	ND (3)
83	TT	54	AA	9.5 ± 2.5 (3)	0.1 ± 0.1 (3)	0.7 ± 0.1 (3)	0.2 ± 0.06 (3)
85	TNA	34	AA	27.4 ± 3.3 (3)	1.4 ± 1.3 (3)	0.6 ± 0.2 (3)	NA
86	TT	35	CA	12.4 ± 2.5 (3)	ND (3)	3.1 ± 2.2 (3)	NA

[a]Tissue from: RM = reduction mammoplasty; NTB = mastectomy of non-tumorous breast; TNA = tumorous breast, normal adjacent tissue; T = tumor tissue, adenocarcinoma.
[b]CA = caucasian; AA = African-American.
[c]RAL = relative adduct labeling in units of adducts formed per 10^6 nucleotides/mg protein/30 min.
[d]Mean plus and minus standard error; N = number of protein samples assayed.
[e]Assays did not distinguish between L-proyl-tRNA synthetase activation or kinase activation, see text.
[f]Prostaglandin H synthase activation, i.e., arachidonic acid-dependent and 100% inhibited by 10 μM indomethacin[7].
[g]ND = not detected.
[h]NA = not assayed due to insufficient protein.

but has never been well characterized. Kato and Yamazoe (11) proposed that L-proyl-tRNA synthetase could esterify N-hydroxy heterocyclic amines and that ATP was required to add the amino acid to the acceptor tRNA via tRNA synthetase. Davis et al. (35) subsequently showed ATP-dependent cytosolic activation of N-hydroxylamines of IQ, MeIQx, and PhIP by organs of monkeys and rats could proceed in the absence of L-proline and termed the ATP-dependent activity a "phosphatase". We have now examined over 32 different human breast tissue samples, and our results so far indicate that cytosolic tRNA synthetases do not participate to a significant extent in the biotransformation of the N-hydroxylamine metabolite of PhIP because in no case has activation been observed with L-proline present and ATP absent. On the other hand, N-OH-PhIP generated DNA adducts with ATP alone. Furthermore, adduct levels were the same or higher with ATP alone than with ATP and L-proline together. To completely rule out tRNA synthetase activation, however, the cytosolic fraction would need to be depleted of endogenous amino acids. To test this notion, tRNA synthetase substrates were removed by dialysis in one experiment, and activation was achieved by adding ATP alone. In other experiments, RNase A was added to digest tRNAs present in cytosolic preparations. The RNase-treatment did not eliminate the ATP-dependent activation of human mammary tissue cytosol. N-OH-PhIP was not activated if the cytosol fraction was boiled, indicating an enzyme-mediated reaction. Furthermore, γ-S-ATP inhibited this reaction. Therefore, it appears that any observed tRNA synthetase activation shown in Table I can be attributed to the ATP present in this assay and suggest the involvement of a kinase or kinases in N-OH-PhIP metabolism. Lin et al. (50) have reached a similar conclusion regarding tRNA synthetase/kinase activation of N-OH-PhIP by human liver cytosol.

We examined breast tissue cytosol from reduction mammoplasty patients for kinase activity and compared the results to those obtained with cytosol from cancer patients. The results are shown in Table 2. Approximately half (11/21) of the breast reduction patients displayed detectable kinase activity. Eight out of these eleven patients had no premalignant conditions such as fibrocystic (FC) lesions. In contrast to reduction mammoplasty, all 12 tissues from breast cancer patients had cytosolic kinase activity, whether the tissue itself was tumorous or was normal tissue adjacent to tumor or tissue from the non-tumorous breast. In addition, the kinase activity for cancer patients was on average much higher than positive breast reduction patients. The data in Table 2 superficially suggest that there is a correlation between kinase activation of cooked meat mutagens and mammary gland tissues from cancer patients. However, the significance of the results is uncertain because of the limited number of individuals involved, especially the smaller number of cancer patients and tumor tissue samples. The present sample of cancer patients was very skewed toward older African-Americans, whereas the breast reduction patients were predominantly younger caucasians. The strength of this tentative association between kinase xenobiotic activity and breast cancer will be tested as more data are obtained.

Cellular signaling pathways in which kinases are activated in transformed cells is one possible explanation for the above correlation. In searching the literature for a kinase that might be capable of phosphorylating N-hydroxy xenobiotics, the tyrosine-specific protein kinase $pp60^{c\text{-}src}$ appears to be a candidate enzyme. $pp60^{c\text{-}src}$ has an extraordinarily broad active site substrate specificity (57); it will phosphorylate a variety of achiral residues attached to peptides and an assortment of free aromatic and aliphatic alcohols. It has been reported that most primary human breast tumors show elevated $pp60^{c\text{-}src}$ activity (58). Indeed, results have been presented that $pp60^{c\text{-}src}$ is involved with two major signaling pathways in human breast cancer (epidermal growth factor receptor-EGFR and $p185^{HER2/new}$) and may contribute to malignant transformation (59). Reports have shown that $pp60^{c\text{-}src}$ protein tyrosine phosphorylation is stimulated by estrogen and such stimulation can lead to cell proliferation in vitro (60). This latter result might be relevant to the kinase activity we detected in breast reduction patients. We hope to be able to ask whether $pp60^{c\text{-}src}$ or other kinases participate in activating the food mutagen PhIP. On the one hand, kinases can be purchased and tested directly for their ability to phosphorylate N-OH-PhIP. Alternatively, kinase inhibitors with specificity against $pp60^{c\text{-}src}$ and anti-$pp60^{c\text{-}src}$ antibodies could be used in an attempt to block N-hydroxy PhIP activation. The same or similar strategy could

Table 2. A Comparison of N-OH-PhIP Kinase Activities in Breast Tissue from Reduction Mammoplasty Patients and Cancer Patients.

Donor No.	Age	Ethnic[a] Origin	RAL [b]	Kinase Activity[c]	Pathology[d]
Reduction mammoplasty patients:					
29	23	AA	26.3	H	ND
58	31	CA	1.5	I	ND
31	29	CA	0.6	L	ND
28	22	CA	0.6	L	FC
49	26	CA	0.4	L	ND
39	29	CA	0.4	L	ND
35	48	CA	0.4	L	ND
33	21	AA	0.3	L	ND
34	21	AN	0.2	L	F
74	23	CA	0.2	L	ND
46	48	CA	0.1	N	FC
48	22	CA	0.1	N	F
21	39	CA	0.0	N	ND
26	37	CA	0.0	N	FC
32	17	CA	0.0	N	ND
36	21	CA	0.0	N	F
42	25	CA	0.0	N	FA
47	36	CA	0.0	N	FC
55	21	CA	0.0	N	F
59	20	AA	0.0	N	ND
71	23	CA	0.0	N	ND
Cancer patients:					
25*	58	AA	26.3	H	NTB
57	73	AA	12.8	H	TT
24*	58	AA	12.1	H	NAT
27	48	CA	4.0	I	NAT
86	35	CA	3.1	I	TT
30	50	CA	2.6	I	NAT
83	54	AA	0.7	L	TT
85	34	AA	0.6	L	TT
37	84	AA	0.6	L	NAT
77	37	AA	0.5	L	TT
80	35	CA	0.3	L	TT
78	41	AA	0.2	L	TT

[a]CA = Caucasian; AA = African-American; AN = Asian-American.

[b]Relative Adduct Labeling expressed as adducts/10^6 nucleotides/mg protein/30 min.

[c]Kinase activity <RAL> classification: N = not present at a level greater than 1 x 10^{-7}; L = low activity (less than 10^{-6}); I = intermediate activity (between 10^{-6} and 10^{-5}); H = high activity (greater than 10^{-5}).

[d]ND = none detected; F = fibrosis; FC = fibrocystic; FA = fibroadenoma; NTB = non-tumorous breast; NAT = normal adjacent tissue; TT = tumor tissue (infiltrating adenocarcinoma).

*Numbers 24 and 25 were tissue from different breasts of the same individual.

be used for other kinases. Cytosol from kinase-positive cancer patients donor numbers 78 and 83 were tested for kinase inhibition with the protein tyrosine kinase inhibitor tyrphostin A23 and the more potent general protein kinase inhibitor K252a (82). In *in vitro* assays, 50 μM tyrphostin A23 inhibited ATP-dependent adduct formation by 84% and 95%, whereas 1 μM of K252a inhibited the same preparations 78% and 64%. (K252a inhibits most serine/threonine kinases with IC_{50}s in the nanomolar range.) These data support the notion that a tyrosine protein kinase is responsible for most of the esterification of N-OH-PhIP.

Human Mammary Epithelial Cell Lines Exhibit Cytosolic Enzymes that Activate the Proximal Dietary Mutagen N-Hydroxy-PhIP to DNA Binding Species.

To dissect the steps and mechanisms of heterocyclic amine genotoxicity, it will be important to have a relevant target cell system in which experimental variables can be controlled, and primary cultures of mammary gland epithelial cells are the obvious choice. However, one problem associated with primary cultures of human cells is routinely obtaining sufficient human mammary epithelial cells from surgical specimens. To circumvent this problem, normal HMEC can be grown and expanded in culture to provide sufficient numbers of cells to study xenobiotic metabolism. Alternately, many investigators have used human breast cell lines to study the biochemistry and molecular biology of human mammary epithelial cells with good success. We have analyzed MCF-7 and MCF-10A human breast cell lines derived from tumor and normal cells, respectively, for phase I and II activation systems. MCF-7 is human breast carcinoma cell line that is widely used in research. MCF-7 has retained many characteristics of differentiated mammary epithelium, such as the ability to respond to and process estradiol via cytoplasmic estrogen receptors (80). In contrast, MCF-10A is a normal breast cell culture that spontaneously immortalized and which grows in monolayers but does not form colonies in soft agar (81).

To validate the use of cell lines as a model for normal HMEC, we first compared food mutagen DNA damage induction between breast cell lines and normal HMEC. The normal HMEC were primary cultures initiated in this laboratory. Cultured cells were exposed to either 20 μM N-OH-PhIP or 100 μM PhIP for 2 h and then assayed for PhIP-DNA adducts by ^{32}P-postlabeling. The results showed that the 2 cell lines metabolized N-OH-PhIP such that the same 3 PhIP-DNA adducts were produced in the same relative proportion as were formed in normal HMEC in culture (data not shown). Relative adduct labeling indices was also similar between the primary cell cultures and cell lines with RAL x 10^{-7} values of: HMEC (log 61) 14.5 $\pm$ 4.6; HMEC (log 70) 20.7 $\pm$ 6.3; MCF-7 cells 25.9 $\pm$ 2.0; and MCF-10A cells 13.6 $\pm$ 3.8. The results therefore demonstrated that these cell lines retained phase II activation pathways for N-OH-PhIP. In agreement with studies using normal HMEC, no adducts were detected following a 2 h exposure to 100 μM PhIP, suggesting that little or no phase I activation occurred in the MCF-7 and MCF-10A cell lines. To determine which cytosolic phase II activation enzymes the breast epithelial cell lines possessed, we next assayed for N-OH-PhIP esterification via acetyltransferases, sulfotransferases and kinases. The results are presented in Figure 4. 87NTB cells are normal HMEC originating from non-tumorous breast tissue of a 57 year old black woman. Compared to the human breast tissue data summarized in Table 1, MCF-7 cells are low N-acetyltransferase/high kinase phase II activity with N-OH-PhIP and no detectable sulfotransferase activity; whereas, MCF-10A cells are intermediate N-acetyltransferase/intermediate kinase activity with slight sulfotransferase activity, and 87NTB cells are high N-acetyltransferase/low kinase with very low sulfotransferase activity. The significantly higher kinase activity associated with MCF-7 carcinoma cells is consistent with our data showing higher kinase levels in mammary gland tissue of tumor patients (see Table 2). MCF-7 and MCF-10A cells also activated proximal mutagen by microsomal PHS (not shown). Therefore, these cell lines some (MCF-7) or all (MCF-10A) of the cytosolic/microsomal phase II activation enzymes as normal HMEC. Based on the data generated so far, MCF-7 appears to be an appropriate example for breast tumor tissue, and MCF-10A appears to be a suitable model to study human mammary gland cell

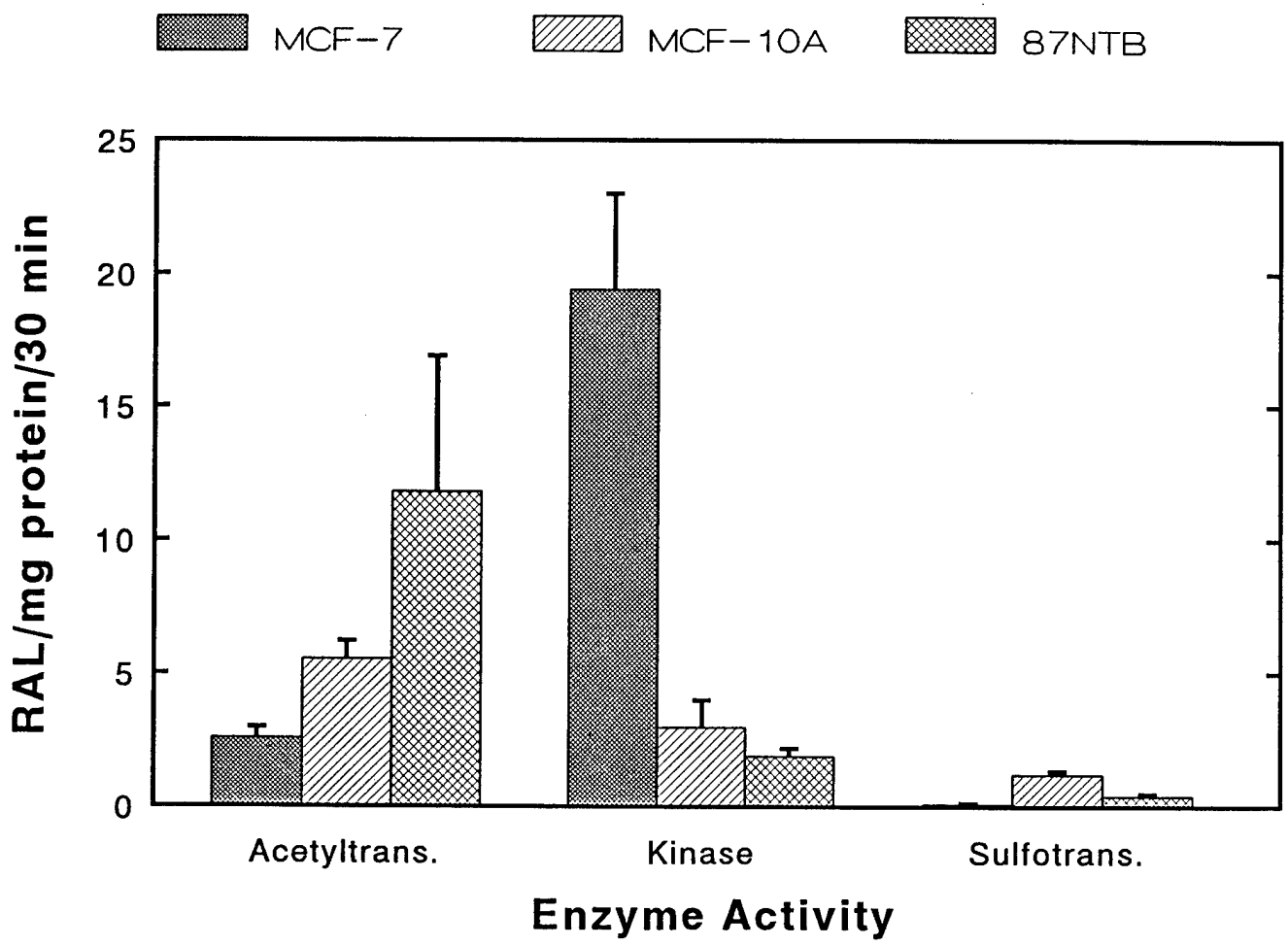

FIGURE 4. Cytosolic acetyltransferase, sulfotransferase, and kinase enzymes mediating N-OH-PhIP binding to DNA. Enzyme activities from the cytosols of human breast cell lines MCF-7 and MCF-10A and primary mammary epithelial cell cultures 87NTB were assayed as described (50). Results are expressed as relative adduct labeling (RAL) per 10^6 nucleotides formed/mg protein/30 min.

heterocyclic amine metabolism, genotoxicity, and carcinogenicity.

Species Differences in the Bioactivation of N-OH-PhIP by Breast Tissue Cytosols from Humans, Rats, and Mice.

In an effort to find a rodent model that would mimic the cooked meat mutagen activation complexity of human breast cells, we assayed mammary gland cytosols obtained from 2-day postpartum, lactating Fischer 344 rats and BALB/C mice. In agreement with Ghoshal, et al. (56) who studied immature, Sprague-Dawley female rats, we found that rat mammary epithelial cells use acetyltransferase almost exclusively to activate N-OH-PhIP. Fischer 344 data (expressed in PhIP adducts/nucleotide/mg protein/30 min) were: acetyltransferase, $<RAL> = 8.8 \pm 1.1 \times 10^{-6}$; sulfotransferase, $<RAL> = 0.4 \times 10^{-8}$; tRNA synthetase/kinase, none detected. The acetyl CoA-dependent enzyme activity in rats was intermediate to that for human rapid and slow acetylator types listed in Table 1. Mice showed more routes of activation with significant kinase and sulfotransferase catalyzed PhIP-DNA adducts in addition to acetyltransferase activity (data not shown). Due to limited mouse mammary gland tissue in these experiments, only single assays and no control reactions were performed. Therefore, these latter experiments will need to be repeated. If these 3 cytosolic activation pathways are indeed present in mouse mammary tissue, then this rodent might be a more suitable model of breast tissue activation pathways of humans. Phase II activities, of course, can vary between murine strains as well as vary with age, sex, nutritional status, etc. We have not yet assayed for microsomal prostaglandin S synthetase activity in the rodent mammary gland samples due to insufficient protein yields.

Cytotoxicity and Genotoxicity Studies: DNA Adducts as Functions of Mutagen Dose and Exposure Time

To determine cellular damage caused by exposure to N-OH-PhIP, we used an enzyme assay for lactate dehydrogenase (LDH) to measure leakage of proteins into the medium and thus, the integrity of the cellular membrane. Data not presented showed that LDH leakage increased over background beginning at 10 μM and was proportional to mutagen dose thereafter. In agreement with the cytotoxicity data, the amounts of DNA-PhIP adducts produced were linear from 2.5 μM to 20 μM N-OH-PhIP. From these results, it appeared that the 20 μM dose was at the threshold of being overtly cytotoxic to human breast cells. We therefore decided to use a 5 μM dose for future studies.

To determine a standard ^{32}P-postlabeling interval, human mammary epithelial cells were treated with 5 μM N-OH-PhIP for different times and DNA-adduct levels were quantified. The time course of adduct accumulation appeared to approximately linear for 8 h (Figure 5). Adduct levels declined after 8 h. Such a decrease in DNA damage represents several variables, such as decomposition of N-OH-PhIP ($t_{1/2}$=10-12 h), removal of lesions via DNA repair and possible intracellular inactivation of mutagen processing enzymes. Therefore to minimize the effects of mutagen instability, enzyme inactivation and DNA repair, a relatively brief exposure time is desirable. On the basis of these experiments and previous work done others (8, 29-35), a standard protocol of a 2 h mutagen exposure was adopted.

Determination of Human Mammary Epithelial Cell DNA Repair Potential.

To obtain information about DNA repair potential in cultured human mammary epithelial cells, MCF-7 cells were treated with 5 μM N-OH-PhIP for 2 h, and DNA was isolated immediately or 8, 24 and 48 h later. As shown in Figure 6, ^{32}P-postlabeling analysis indicated that 80% of the induced adducts were removed by 24 h and 90% were removed by 48 h. These results agree well with most DNA repair data of human cells gown in culture. However, mammary epithelial cell DNA repair efficiency is not as good as for human fibroblasts wherein more than 90% of adducts are removed by 24 h (83). As with all studies reported thus for PhIP was included as a phase I-unactivated control in these experiments. Surprising 200 μM PhIP gave positive results as indicated by the open squares in Figure 6. For the PhIP experiments, we observed no adducts on autoradiographic exposure times

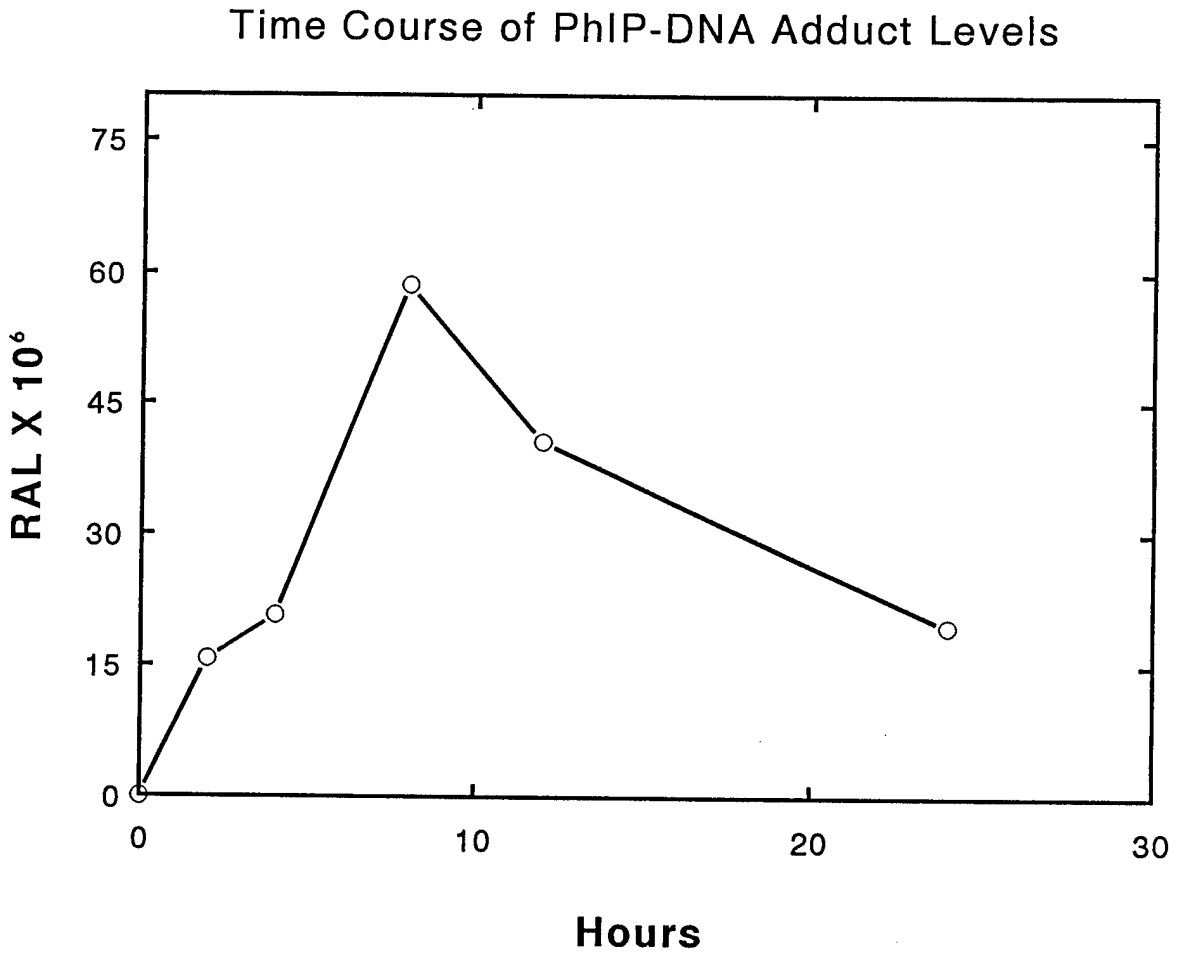

FIGURE 5. Kinetics of PhIP-DNA adduct formation in MCF-7 cells. Cultures of human breast cells were exposed to 5 μM N-OH-PhIP for times varying from 1 to 24 h, and DNA adduct levels were determined by ^{32}P-postlabeling techniques. Results are expressed as adducts per 10^6 nucleotides (RAL). The data are the average of two cultures at each time interval.

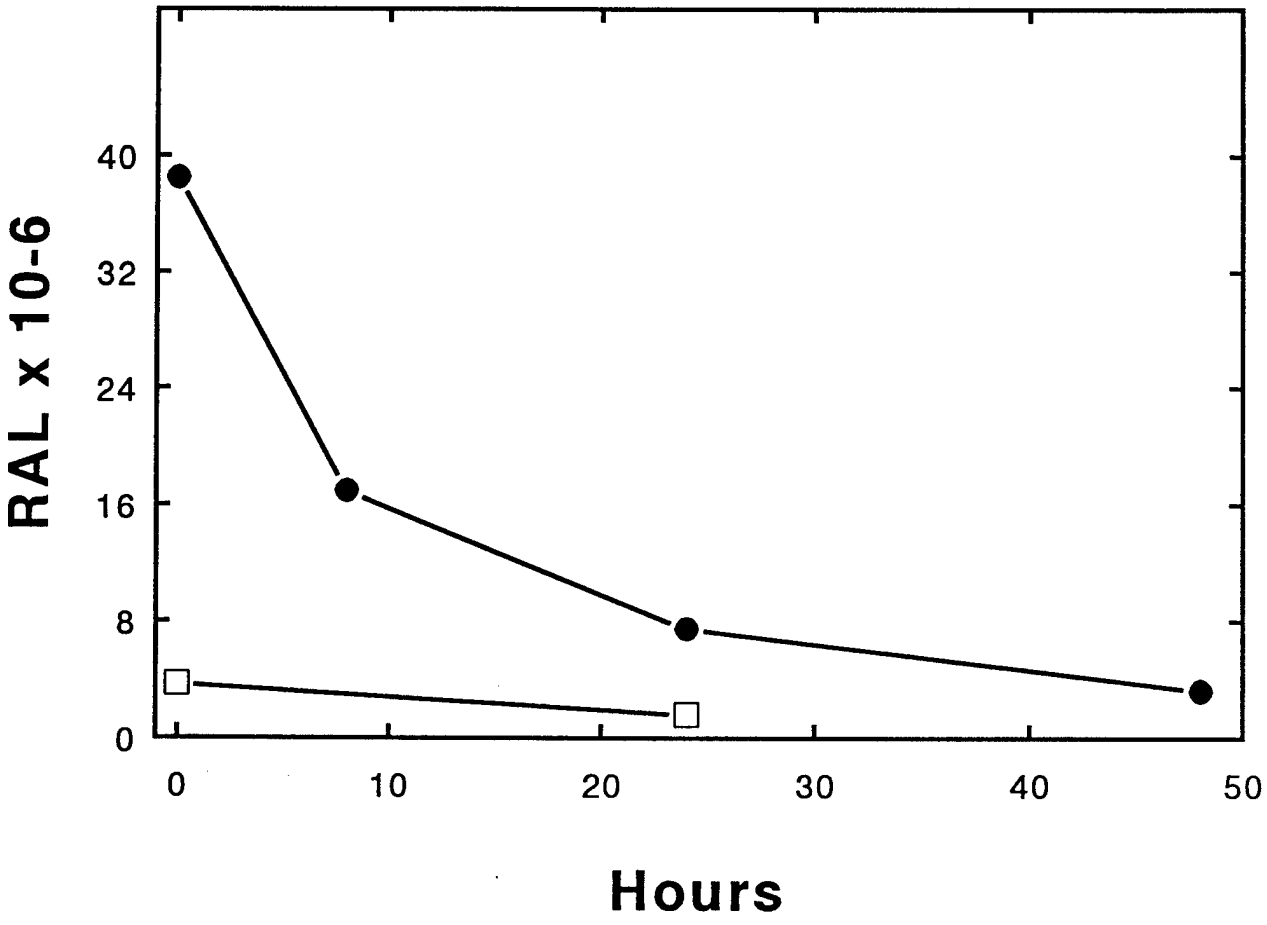

FIGURE 6. Repair of dietary mutagen DNA damage in human breast cells. Cultures of MCF-7 breast cancer cells were exposed to mutagen for 2 h, then mutagen was removed and adduct levels determined as a function of time thereafter. Data are shown as PhIP-DNA adducts per 10^6 nucleotides (RAL) and are the average of 2 experiments. Filled circles, 5 μM N-OH-PhIP mutagen data; open squares, 200 μM PhIP mutagen data.

equivalent to those for 5 μM N-OH-PhIP assays but nonetheless determined cpms in TLC regions where adducts migrate in positive postlabeling. The resultant radioactivity was higher than the background controls, and the results are presented in Figure 6 although they have not been consistently noticed.

Resveratrol, a Natural Product Common in Diets, Inhibits PhIP-DNA Adduct Formation in Cultured Human Breast Cells.

It was reported last year that resveratrol (a phytochemical found in many plant species) inhibited both cyclooxygenase I (COX-1) activity and DMBA-induced preneoplastic lesions in mouse mammary gland (47). These results were interpreted as tumor chemopreventive activity at the stages of initiation and promotion, respectively. Since our preliminary studies demonstrated that a COX-1 activity could activate N-OH-PhIP, we were interested to learn if resveratrol could inhibit PhIP-DNA adduct formation in human mammary epithelial cells. Therefore, we investigated the in vitro effects of resveratrol on primary cultures of human ductal mammary epithelial cells. A dose of resveratrol was selected for use based on published values that showed maximal inhibition of COX-1 at 50 μM and proliferation of mammary gland preneoplastic lesions were inhibited approximately 50% by the 50 μM dose (47). As shown in Figure 7, resveratrol inhibited PhIP-DNA induction in vitro. The results from 3 experiments are presented in Table 3. Resveratrol inhibited PhIP-DNA adduct formation 31%, 43% and 69% in cultured human breast cells. Thus, inhibition levels differed between individuals. The observed variance may be related to the different activity patterns of the phase II pathways discussed previously. From these preliminary studies, we conclude that resveratrol may have chemopreventive properties against human breast cancer, but the 50 μM concentration is more pharmacological than physiological. Other preliminary data not shown indicated that 2.5 μM resveratrol inhibited adduct levels as effectively as 50 μM in one experiment, 40.0% versus 42.8%, respectively, which makes the result more meaningful.

Resveratrol Inhibits Acetyltransferase and Sulfotransferase Phase II Carcinogen Activation Enzymes.

The observed decrease in adduct levels might be due to resveratrol inhibiting mutagen phase II activation. So experiments were performed to analyze the effects of resveratrol on the carcinogen activation pathway described above. Since humans exhibit variable levels of enzyme activities, mouse liver extracts were included as positive controls because mouse liver has relatively high levels of the cytosolic phase II enzymes (50). The results are shown in Figure 8. Resveratrol inhibited human mammary gland and mouse liver acetyltransferases and sulfotransferases. Resveratrol had the greatest effect on sulfotransferase activity; essentially totally suppressing mouse liver sulfotransferase-mediated adduct formation while inhibiting human breast enzymes by approximately 70%. The effect of resveratrol on acetyltransferase activation of N-OH-PhIP was again greater for mouse liver than mammary gland enzymes, 75% inhibition versus 30% to 60% inhibition, respectively. In data not shown, mouse liver kinase activation was strongly inhibited by resveratrol (82%), whereas human mammary gland kinase activation was stimulated by 25% to 28%. This latter results demonstrates that resveratrol inhibition is not due to direct inactivation of mutagen or to inhibition of cellular uptake of mutagen, but resveratrol is instead a enzyme-specific inhibitor of carcinogen phase II enzymes which is likely the mechanism of resveratrol action.

Address to Statement of Work

III-B.1.d. **Statement of Work**

Objective 1: **The cellular uptake and binding of mutagen to protein, RNA, and DNA will be characterized with radiolabeled heterocyclic amines; metabolic processing of heterocyclic amines will be characterized by enzyme analysis and enzyme-**

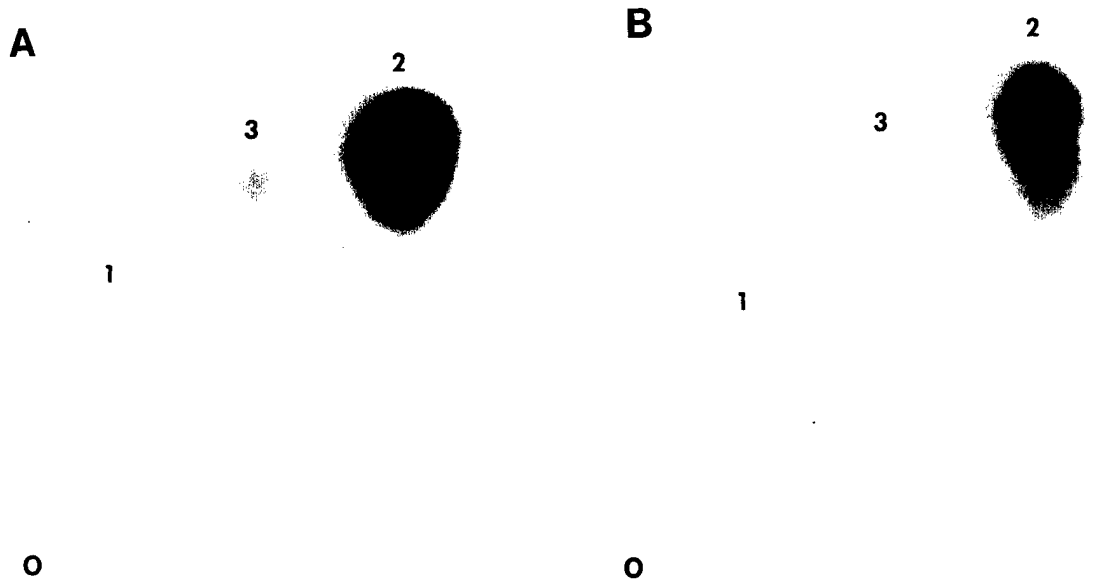

FIGURE 7. Postlabeling maps of PhIP-DNA adducts formed in primary cultures of human mammary epithelial cells when incubated for 2 h with: (A) 20 μM N-OH-PhIP and (B) 20 μM N-OH-PhIP plus 50 μM resveratrol.

Table 3. Effects of resveratrol (RES) on PhIP-DNA adduct formation in primary cultures of human mammary epithelial cells.

Donor Cells[#]	PhIP-DNA Adducts (CPMs)		Percentage Inhibition
	20 μM N-OH-PhIP	20 μM N-OH-PhIP + 50 μM RES	
46 CA	44,652 $\pm$ 4,699	13,916 $\pm$ 1,225	68.8%
18 CA	48,977 $\pm$ 8,827	28,022 $\pm$ 1,836	42.8%
17 CA	65,522 $\pm$ 1,323	42,939 $\pm$ 13,711	31.3%

#Age/Ethnic Origin; epithelial cells were isolated from human mammary gland ductal tissue. Cells were suspended at approximately 10^7 cells/ml in DMEM (supplemented with 1% FBS and 10 mM L-glutamine) and exposed to mutagen or mutagen plus resveratrol for 2 h at 37°C. Cellular DNA was isolated and DNA adducts quantified by ^{32}P-postlabeling.

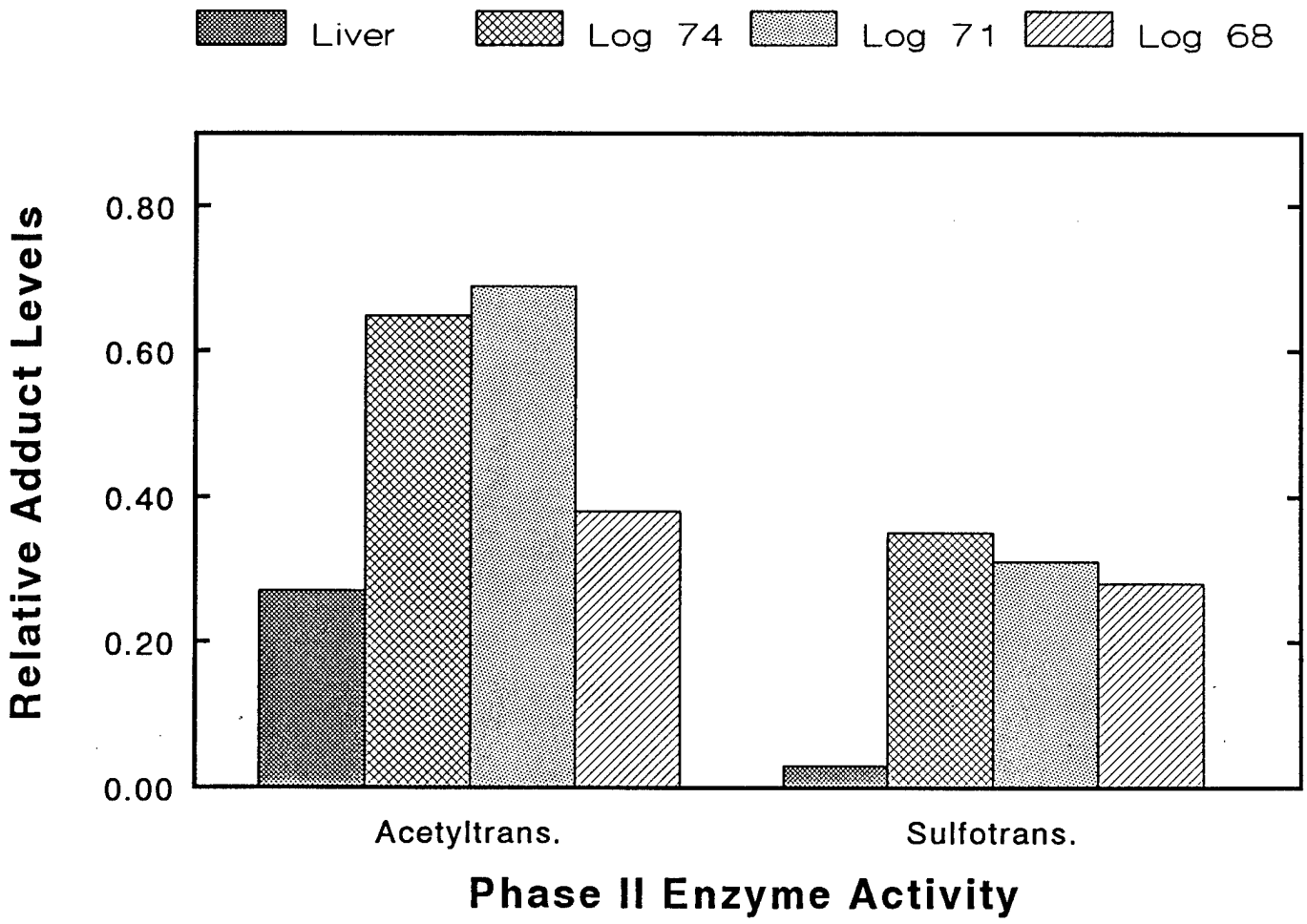

FIGURE 8. Effect of resveratrol on individual phase II activation pathways of acetyltransferase and sulfotransferase from mouse liver and human mammary tissue cytosols. Relative adduct levels are expressed as the ratio of adducts formed in the presence of 50 μM resveratrol to adducts formed in the absence of resveratrol (1.0). Log number refers to different samples and is the same as donor number shown in Table 1. In data not shown, mouse liver kinase activation was strongly inhibited by resveratrol (82%), whereas human mammary kinase activation was somewhat stimulated (25% to 28%).

inhibitor studies; heterocyclic amine metabolites will be characterized by chromatographic techniques.

Task 1: Months 1-3: Chemical syntheses and characterization of N-hydroxy-PhIP and N-hydroxy-MeIQx.

Task 2: Months 2-5: Heterocyclic amine cytotoxicity dose levels established for mammary epithelial cells.

Task 3: Months 6-9: Cellular uptake and macromolecular distribution of covalent binding determined for heterocyclic amines and N-hydroxyl-heterocyclic amines.

Task 4: Months 6-15: Preparation of cytosolic and microsomal fractions of mammary epithelial cells; O-acetyltransferase and sulfotransferase activity in subcellular fractions assayed; enzyme inhibitor studies performed, and heterocyclic amine metabolites identified by chromatographic methods.

Objective 2: **Determination of differential genotoxic effects of heterocyclic amines in target cells: DNA-adducts analyzed as functions of dose and time; relationship between MeIQx and PhIP with their N-hydroxyl metabolites; comparison of induced DNA damage levels and repair efficiency between human and rat mammary epithelial cells.**

Task 1: Months 12-15: Analysis of mammary cell DNA adducts induced by heterocyclic amine exposure; initial damage levels measured; comparison of human and rat mammary epithelial cells.

Task 2: Months 14-18: Genotoxicity studies performed, including quantifying DNA adducts as a function of mutagen dose and exposure time. Comparison of MeIQx, PhIP, N-hydroxyl-MeIQx, and N-hydroxyl-PhIP.

Task 3: Months 16-24: Determination of human and rat mammary epithelial cell DNA repair potential; comparison of MeIQx-DNA and PhIP-DNA adduct removal.

As described above, we were unable to synthesize the N-hydroxylamine proximal carcinogens (Objective 1; Task 1). This in essence eliminated Task 3 because cellular uptake, macromolecular binding studies etc. relied on using radioactive N-OH-heterocyclic amines which can not be purchased (although parent compounds, such as [^{3}H]-PhIP, can be bought.) and thus have to be synthesized in-house. On the other hand, Task 2 and parts of Task 4 of Objective 1 and Tasks 1, 2 and 3 of Objective 2 were done. More importantly other work not listed in the original statement of work was done. Work with more biological significance and health relevance than the original tasks. These studies include the analysis of over 30 individual human breast samples for phase II activation enzymes, the discovery of a xenobiotic kinase(s) possibly associated with human mammary tumors; the metabolic characterization of food mutagen by widely used human breast cell lines; preliminary findings on rat mammary tissue metabolic activation capabilities, and; the demonstration that resveratrol can inhibit N-OH-PhIP induced DNA adducts in primary cultures of ductal epithelial cells and might have chemopreventive effects in retarding the initiation of breast tumors. We have one paper in press and are currently working on two manuscripts with an overall goal of four publication based on this research.

CONCLUSIONS

In conclusion, the human mammary gland has the capacity to metabolically activate the food-derived carcinogen N-OH-PhIP by multiple enzymes systems, including acetyltransferase, sulfotransferase, kinase and prostaglandin hydroperoxidase catalysis. These phase II metabolic mutagen processing enzyme activities vary widely between individuals, and each individual has a unique combination of activation pathways. In vivo, these metabolic processes may play a role in the initiation of breast cancer. Human breast tissue however did exhibit a proline tRNA synthetase activity for esterifying N-OH-PhIP. Alternatively, we discovered that a kinase is capable of activating the mutagen N-OH-PhIP to DNA-binding species and present preliminary kinase characterization data. Furthermore, the data suggest that this xenobiotic kinase activity may be a biomarker for mammary gland tumors. Experiments showed that N-OH-PhIP is cytotoxic and genotoxic to mammary ductal epithelial cells in culture, that the same PhIP-DNA adducts are induced in breast cells as are produced in postlabeling activation assays, and that human mammary gland cells can repair this type of DNA lesion with reasonable efficiency. We conclude that mammary epithelial cells from two strains of rats (Fischer 344 and Sprague-Dawley) activate N-OH-PhIP via acetyltransferase almost exclusively, and therefore may not be good models for cooked-meat-carcinogen metabolism in humans. On the other hand, preliminary data demonstrated that primary cultures of mammary epithelial cells and breast cell lines such as MCF-7 (tumor cells) and MCF-10A (normal cells) appear to be good model systems for the study of mammary cell carcinogen metabolism. We conclude that the dietary phytochemical resveratrol significantly inhibits PhIP-DNA adduct formation in primary cultures of human mammary epithelial cells and that resveratrol inhibits acetyltransferase and sulfotransferase activation of N-OH-PhIP and thus may explain in part its mechanism of action. From these preliminary studies, we conclude that resveratrol may have chemopreventive properties against human breast cancer.

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FINAL REPORT

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Publications

Papers.

Dubuisson, J.G. and Gaubatz, J.W. (1998) Bioactivation of the proximal food mutagen 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine to DNA-binding species by human mammary gland enzymes, Nutrition, 14, in press.

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Bioactivation of the Proximal Food Mutagen 2-Hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (N-OH-PhIP) to DNA-Binding Species by Human Mammary Gland Enzymes

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ABSTRACT

We have investigated phase II activation of the food-derived mutagen 2-hydroxyamino-1-methyl-6-phenyl[4,5-*b*]pyridine (N-OH-PhIP) by cytosolic acetyltransferase, sulfotransferase, and tRNA synthetase/kinase enzymes from human breast tissue. Cytosol from homogenates of mammary gland tissue obtained from breast-reduction surgery or mastectomy was incubated with and without enzyme-specific cofactors, and mutagen binding to calf thymus DNA was quantified by ^{32}P-postlabeling. In addition, microsomal fractions of mammary epithelial cells from some individuals were examined for prostaglandin H synthetase activation of N-OH-PhIP. Our results show that all four enzymes can participate in activating N-OH-PhIP, thus inducing PhIP-DNA adduct formation in human mammary cells. However, not all individuals exhibited all these activities; instead each individual showed a combination of one or more activation pathways. The present findings demonstrate that the human mammary gland has the capacity to metabolically activate a dietary mutagen by several enzyme systems, including acetyltransferase, sulfotransferase, tRNA synthetase/kinase, and prostaglandin hydroperoxidase catalysis. *Nutrition* 1998;14:000–000. ©Elsevier Science Inc. 1998

Key words: dietary carcinogens, human mammary gland, metabolic activation, DNA damage, heterocyclic amines, breast cancer

INTRODUCTION

Mutagenic heterocyclic amines produced during the cooking of meat are one group of dietary compounds that may constitute a risk factor in the etiology of human breast cancer.[1,2] The heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is estimated to be the most abundant promutagenic compound in grilled meats[3] and is a mammary gland carcinogen in rodents.[4] Initiation of carcinogenesis by heterocyclic amine compounds is believed to be due to their adduction to DNA following metabolic activation of the parent amines. The first step in the bioactivation of PhIP is a cytochrome P450-mediated hydroxylation of the amine nitrogen, catalyzed by liver microsomal P450 1A2 in humans.[5] N-hydroxyamino-heterocyclic compounds are relatively non-reactive with DNA under physiologic conditions. To become more reactive and form specific adducts, N-hydroxy-PhIP (N-OH-PhIP) is esterified by one or more enzymes during phase II activation. Esterification of N-OH-PhIP is relatively low in human liver tissue with few PhIP-DNA adducts produced.[6] Therefore, N-OH-PhIP is released systemically for potential phase

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II activation in extrahepatic tissues, such as breast tissue, wherein these activation processes could play a significant role in organ-specific tumorigenesis.

To determine what phase II systems can metabolically process N-OH-PhIP to PhIP-DNA adducts, we have tested for acetyltransferase, sulfotransferase, and aminoacyl-tRNA synthetase/kinase activation by human breast tissue cytosol. Our results show that all three phase II enzyme pathways can participate in esterifying N-OH-PhIP, thereby producing PhIP-DNA adducts. In addition to cytosolic enzymes, microsomal fractions of mammary epithelial cells from some individuals exhibited an arachidonic acid-dependent, indomethacin-inhibited capacity to activate N-OH-PhIP, indicating prostaglandin H synthase (PHS) catalysis as another route of bioactivation.[7] This is the first report to demonstrate the multiplicity of metabolic pathways for N-hydroxy food mutagen activation in human breast tissue.

SUBJECTS AND METHODS

Human mammary gland tissue, removed from healthy women undergoing reduction mammoplasty, was obtained from The Cooperative Human Tissue Network. Residual surgical breast tissue was also obtained following mastectomy from the University of South Alabama Hospitals. Cytosolic and microsomal fractions were prepared by standard differential certifugation.[8] Enzyme assays were performed immediately after cytosol isolation. N-OH-PhIP was supplied by Midwest Research Institute (Kansas City, MO).

Cytosolic activation assays were conducted as previously described.[8] The assay mixtures (0.2 mL) contained 100 mmol potassium phosphate (pH 7.4), 1 mmol DTT, 2 mg/mL calf thymus DNA, 1 mg/mL cytosolic protein, 0.1 mmol EDTA, and 20 μmol N-Oh-PhIP. Enzyme assays and concentrations of enzyme-specific cofactors were: acetyltransferase, 1 mmol acetyl-CoA; sulfotransferase, 0.2 mmol 3′-phosphoadenosine 5′phosphosulfate; and tRNA synthetase/kinase, 1 mmol L-proline and 1 mmol adenosine triphosphate (ATP). The sulfotransferase assay did not contain DDT, and the kinase assay had 50 mmol Bicine (pH 8.0) in place of potassium phosphate. To measure PHS peroxidase activation, assays contained 50 mmol potassium phosphate buffer (pH 7.4), 100 μmol arachidonic acid, 2 mg/mL DNA, 20 μmol N-Oh-PhIP, and 1 mg/mL microsomal protein. In separate reactions, 10 μmol indomethacin was added as an inhibitor of PHS.[7] Enzyme-specific cofactors were absent in negative control assays. Assay mixtures were purged with argon, substrate was added, and mixtures were incubated at 37° for 30 min. The reactions were terminated and modified DNA isolated as described.[8]

^{32}P-postlabeling analysis was performed as reported earlier using the ATP-deficient method.[9] Enzymes and chemicals were from Sigma Chemical Co. (St. Louis, MO). Nucleotides were labeled with T4 polynucleotide kinase and carrier-free [γ-^{32}P]ATP ≥4,000 Ci/mmol (ICN, Costa Mesa, CA). Adducts were separated by thin-layer chromatography (TLC), carried out on polyethyleneimine-cellulose sheets (Alltech Associates, Deerfield, IL, USA) that were developed sequentially in: D1, 1.7 mol/L sodium phosphate (pH 6.0); D2, 3.5 mol/L lithium formate, 6.8 mol/L urea (pH 3.5); D3, 0.6 mol/L Tris-HCl-0.6 mol sodium phosphate, 6.5 mol/L urea (pH 8.2); and D4, 1.7 mol/L sodium phosphate (pH 6.0). PhIP-DNA adducts were detected by autoradiography on x-ray film using intensifying screens at −70°C. Radioactivity associated with an adduct was determined by Cerenkov counting after cutting an area out of a TLC sheet corresponding to an adduct. The same chromatographic areas were removed from negative control reactions (i.e., minus cofactor) for use in background subtractions. The level of covalently modified nucleotide was determined from relative adduct labeling (RAL). RAL was calculated from the formula: RAL = (cpm in adduct − cpm of background)/cpm in total nucleotides, then corrected for standard labeling conditions of ATP excess by the method of Schut and Herzog.[10]

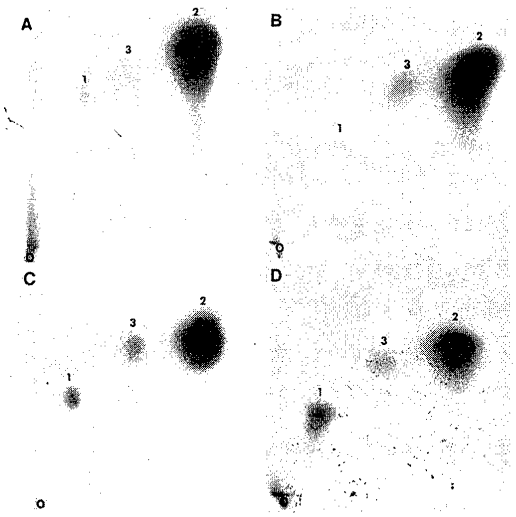

FIG. 1. Autoradiograms showing PhIP-DNA adduct patterns obtained by incubating 20 μmol N-OH-PhIP with 2 mg/mL of calf thymus DNA in the presence of: (A) 1 mmol acetyl CoA; (B) 1 mmol adenosine triphosphate (ATP); (C) 0.2 mmol PAPS; and (D) 100 μmol arachidonic acid. Incubations A, B, and C were with 1 mg/mL cytosolic protein from human breast tissue, and incubation D was with 1 mg/mL of microsomal protein from breast tissue. O is the chromatographic origin. Adducts are assigned number 1, 2, and 3 based on previous studies.[10,11,14,15] Adducts were not observed in negative control reactions without cofactors for matching autoradiography exposure times.

RESULTS

In the studies reported here, the ^{32}P-postlabeling method was used to test the ability of human breast tissue enzymes from six donors to convert the intermediate mutagen N-OH-PhIP to PhIP-DNA adducts. As shown in Figure 1, this in vitro phase II metabolic activation of N-OH-PhIP was measured by the binding of PhIP to DNA in the presence of human breast tissue cytosolic or microsomal proteins and specific cofactors for acetyltransferase (Fig. 1A), sulfotransferase (Fig. 1B), tRNA synthetase/kinase (Fig. 1C), and PHS (Fig. 1D). The individual mutagen activation results are summarized in Table I. The (date) indicate that human mammary gland has the capacity to metabolically activate N-hydroxy food mutagens through at least four different enzyme pathways. Not all individuals possessed all of the activities, but instead each donor showed a variable combination of one or more of the activities. For example, donor number 25 showed N-OH-PhIP activation via all four esterification reactions, whereas only acetyltransferase and PHS peroxidase from donor number 26 activated the mutagen. Thus, each individual exhibited a unique activation profile.

Three PhIP-DNA adducts are induced in cultured human mammary epithelial cells exposed to N-OH-PhIP[11] (data not shown). To validate the in vitro system used here, we compared adduct patterns generated by primary cultures of mammary epithelial

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TABLE I.

N-HYDROXY-PHIP BINDING TO DNA MEDIATED BY HUMAN BREAST TISSUE PHASE II ACTIVATING ENZYMES

Donor number	Tissue source*	Age	Ethnic origin†	PhIP-DNA adducts (RAL × 10^{-6})‡ Mean ± SE (N)			
				Acetyltransferase	Sulfotransferase	tRNA Synthetase/Kinase§	Peroxidase‖
21	RM	39	CA	33.0 ± 4.7 (3)	1.2 ± 0.2 (2)	ND (2)	NA
25	NTB	58	AA	1.6 ± 0.3 (2)	2.3 ± 0.8 (2)	21.6 ± 3.5 (2)	1.5 ± 4.1 (2)
26	RM	37	CA	20.6 ± 6.3 (3)	ND (3)	ND (3)	1.6 ± 0.3 (2)
27	TNA	48	CA	1.3 (1)	0.5 (1)	3.7 (1)	NA
28	RM	22	CA	17.8 ± 2.7 (3)	0.7 ± 0.2 (3)	ND (1)	ND (1)
29	RM	33	AA	ND (2)	NA	26.3 ± 5.0 (2)	NA

* Tissue from: NTB, mastectomy of non-tumorous breast; RM, reduction mammoplasty; TNA, tumorous breast, normal adjacent tissue.
† AA, African-American; CA, caucasian.
‡ RAL, relative adduct labeling in units of adducts formed per 10^6 nucleotides/mg protein/30 min (see text for details of quantification).
§ Assays did not distinguish between L-proyl-tRNA synthetase activation or kinase activation (see text).
‖ Prostaglandin H synthase activation, i.e., arachidonic acid-dependent and 100% inhibited by 10 μmol indometacin.[7]
NA, not assayed due to insufficient protein; ND, not detected.

cells and by the enzyme assays. The same three PhIP-DNA adducts were also observed via ^{32}P-postlabeling following all esterification assays that resulted in DNA binding (Fig. 1). We conclude that these in vitro esterification activities are capable of producing DNA adducts in vivo. In addition, we repeatedly failed to detect adducts generated with the parent compound PhIP (20 μmol) either in the enzyme assays or cultured cells. This latter result suggests that PhIP phase I enzymes are absent or very low in mammary epithelial cells. The absence of PhIP activation may reflect substrate specificity, however, because Pfau et al.[12] have presented evidence that the unmodified heterocyclic amines 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-*f*] quinoline (MeIQ) can be metabolically activated to DNA binding species by human mammary epithelial cells in culture.

DISCUSSION

The results in Table I suggest that acetyltransferase and tRNA synthetase/kinase are major pathways of phase II activation, whereas sulfotransferases and PHS play less prominent roles in N-OH-PhIP esterifcation. Of the four phase II transferases in human breast tissue, acetyltransferase and tRNA synthetase/kinase had the highest capacity to induce PhIP-DNA adducts. Positive results of sulfotransferase and PHS peroxidase gave uniformly lower levels of PhIP-DNA adducts, ranging from 0.5×10^{-6} to 2.3×10^{-6} adducts/nucleotide. Five out of six samples had detectable acetyltransferase activity; three with higher levels of PhIP-DNA adducts and two with lower levels. We assume that this grouping corresponds to the bimodal distribution of rapid and slow acetylators representing the polymorphic N-acetyltransferase loci of the human population[13] with the not detected sample being on the lower end of the slow acetylators. Sadrieh et al.[14] have observed a similar distribution of N-acetyltransferase expression and activity in the human mammary gland. In contrast to the acetyltransferase results, only half of the samples showed tRNA synthetase/kinase activity. It was proposed that L-proyl-tRNA synthetase could esterify N-hydroxy heterocyclic amines[15] and that ATP was required to add the amino acid to the acceptor tRNA via tRNA synthetase. Our results so far indicate that cytosolic tRNA synthetases do not participate to a significant extent in the biotransformation of the N-hydroxylamine metabolite of PhIP because no activation was noted in the presence of L-proline but in the absence of ATP. Conversely, activation was observed in the absence of L-proline and the presence of ATP. Therefore, it appears that any observed tRNA synthetase activation could be attributed to the ATP present in this assay and suggest the involvement of a kinase in N-OH-PhIP metabolism. Lin et al.[8] have reached a similar conclusion regarding tRNA synthetase/kinase activation of N-OH-PhIP by human liver cytosol. To completely rule out tRNA synthetase activation, however, the cytosolic fraction would need to be depleted of endogenous amino acids, and we have not performed these experiments. Therefore, we can not say with certainty whether this activity is due to a kinase.

The data shown in Table I indicate a diversity of N-OH-PhIP activation enzymes in human breast tissue not observed in rat mammary glands. In agreement with Ghoshal et al.[16] who studied Sprague-Dawley female rates, we have found that Fischer 344 rat mammary gland epithelial cells use acetyltransferase to activate N-OH-PhIP but have no detectable kinase or sulfotransferase activity (data not shown). By contrast, individual human phase II activation patterns ranged from high acetyltransferase activity, low sulfotransferase activity, and no detectable kinase activity to high kinase activity and low acetyltransferase and sulfotransferase activities. Therefore, human mammary gland cells possess multiple N-OH-PhIP activation systems that display highly variable interindividual levels of mutagen processing enzyme activities. Additional studies will be required to determine if any of these enzyme activities, or combination of activation pathways, constitute a risk factor for mammary tumors in humans.

SUMMARY

In conclusion, the human mammary gland has the capacity to metabolically activate N-OH-PhIP by several enzyme systems, including acetyltransferase, sulfotransferase, kinase, and prostaglandin hydroperoxidase catalysis. In vivo, such processes may play a role in the initiation of breast cancer.

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